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Tracheal Function During Influenza Infections

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Studies with animal models have demonstrated that viral respiratory tract infections suppress bacterial clearance processes in the lung and that this alteration in host defenses appears to explain the excessive mortality from bacterial pneumonia during influenza epidemics. However, since the pathogenesis of postinfluenza pneumonia and other pneumonias probably involves the aspiration of normal nasopharyngeal flora, injury to major airways associated with influenza infections could also contribute to the development of bacterial pneumonia by increasing bacterial deposition in the peripheral lung. We investigated this possibility by evaluating tracheal clearance processes and spontaneous changes in the tracheal flora in a murine model for acute influenza. During fine-particle aerosol exposures to *Staphylococcus aureus*, influenza-infected mice retained the same number of bacteria on their proximal tracheal surfaces as did control mice, and the relative adherence of the staphylococci to the trachea was similar in both groups of mice. However, the clearance of viable staphylococci from the trachea was significantly delayed in influenza-infected mice. Control and influenza-infected mice were also evaluated for changes in their normal tracheal flora. Mice with established influenza infections had more frequent spontaneous colonization with gram-negative bacteria, more bacterial isolates per animal, and higher bacterial concentrations in tracheal homogenates than control mice. These changes in tracheal flora were most pronounced on day 7 after virus inoculation and persisted after virus titers were undetectable, but eventually resolved by day 14 after virus infection. Tetracycline therapy started 2 days after virus inoculation prevented the increased colonization. This impaired clearance function and increased spontaneous colonization were associated with morphological evidence of mucosal regeneration. We conclude that spontaneous changes in tracheal flora occur during influenza infections, that these changes reflect, in part, impaired clearance functions, and that such changes could contribute to the development of pneumonia regardless of the clearance capacity of the lung parenchyma.

Influenza epidemics are associated with excessive mortality from both bacterial pneumonia and chronic nonspecific lung disease (1, 5). Since these viruses primarily replicate in the nasopharynx and major airways, the pathogenesis of acute respiratory failure in patients with established chronic lung disease seems relatively obvious. However, the relationship between viral respiratory tract infections and bacterial superinfections is less clear. In general, viral infections could alter the balance between regional defense mechanisms in the lung and bacterial growth either by suppressing bacterial clearance processes or by increasing bacterial deposition in the peripheral airways and pulmonary parenchyma. Since Jakab and Green demonstrated that resolving paramyxovirus (Sendai) infections impair the intrapulmonary killing of *Staphylococcus aureus*, it has been generally agreed that the primary effect of viral infections is suppresion of bacterial clearance (9, 10).

However, we have observed that the pattern of bacterial deposition during aerosol challenges changes in experimental influenza infections and that a larger fraction of the staphylococci delivered to the lower respiratory tract is located in the major airways of influenza-infected mice (13). This alteration suggests that viral tracheobronchitis increases tracheobronchial susceptibility to colonization, impairs tracheobronchial clearance processes, or alters airflow patterns which influence particle deposition. If similar bacterial accumulation on the tracheobronchial mucosa occurred spontaneously after minor aspirations during viral infection, then virus-induced airway disease could contribute to the

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development of bacterial superinfections, even though the changes in physical transport from the lung probably do not contribute to the defect in bacterial clearance observed during viral infections (9). For example, the presence of microfoci of bacteria on the surface of airways could easily change the pattern or intensity of bacterial deposition into the peripheral lung. To further evaluate tracheal function during viral tracheobronchitis, we measured tracheal clearance processes and spontaneous changes in the tracheal microflora during acute influenza infections in mice.

MATERIALS AND METHODS

Mice. Healthy female CF1 mice were used throughout these experiments.

Virus infection procedure. The virus stock (influenza A/PR8/34) used for these studies had been serially passed in CF1 mice to increase virulence (11). Mice were lightly anesthetized with ether and inoculated with 0.05 ml of virus intranasally. The dose used was determined with pilot studies and produced obvious clinical illness in more than 90% of the mice and approximately 10% mortality by 14 days.

Viral titration. Madin-Darby canine kidney cells (Flow Laboratories) were maintained and propagated in Eagle minimum essential medium with 5% heat-inactivated newborn calf serum and antibiotics by using Linbro tissue culture plates (2-cm^2 surface area per well). Tracheal homogenates were serially diluted in tissue culture medium containing 1% bovine serum albumin (fraction V; Sigma Chemical Co.) and penicillin (1,000 U/ml), and portions of these dilutions (0.2 ml) were then layered on confluent monolayers of Madin-Darby canine kidney cells. After a 1-h absorption period, 1.3 ml of medium was added to each well, and monolayers were incubated for 48 h. Virus released into the tissue culture medium was identified with a hemagglutination microtiter technique (O+ human erythrocytes), and the tissue culture infectious dose 50% titer was calculated by the Reed-Muench approximation (17). Virus release was associated with obvious cytopathic effect (monitored with phase microscopy).

Staphylococcal aerosol experiments. (i) Tracheal clearance measurements. Control and influenza-infected (day 7) mice were exposed to fine-particle aerosols of *S. aureus* 502A by techniques previously described in detail (12). After aerosol exposure, mice were sacrificed and the proximal trachea (segment above thoracic inlet) was removed aseptically. These tracheal sections were homogenized in 1 ml of sterile phosphate-buffered saline (pH 7.4) with a glass mortar and pestle, and the homogenates were diluted and plated in duplicate (100-λ aliquots). The number of viable staphylococci in each trachea removed 3 or 6 h after aerosol exposure was normalized to the mean number of viable staphylococci in the tracheas removed from the mice sacrificed immediately after aerosol exposure. These residual fractions were averaged and plotted against time to determine clearance.

(ii) Tracheal adherence after aerosol exposure. After the aerosol exposure described above, the tracheas of some mice sacrificed immediately after exposure were lavaged with sterile phosphate-buffered saline (pH 7.4). We lavaged the tracheas by securing the mice to an animal surgical board, exposing the trachea and transecting it at the thoracic inlet, and slowly injecting 1.0 ml of buffered saline at the proximal end of the trachea through a 27-gauge needle. These washed tracheas were then homogenized and cultured in duplicate by the procedure described above. The number of viable staphylococci in each washed trachea was then normalized to the mean number of staphylococci in unwashed tracheas. These adherent fractions were then averaged to compare the relative adherence in control and influenza-infected mice.

Spontaneous tracheal colonization. (i) Animal groups. Two days after virus inoculation, mice were randomly distributed into an untreated influenza group, a tetracycline-treated group (1 g/liter of drinking water; approximate dose, 60 mg/kg per day), and an amantadine-treated group (0.1 g/liter of drinking water). Control mice received diluent intranasally and ingested untreated drinking water throughout these experiments. On days 1, 5, 7, 10, 14, and 21 after virus or diluent inoculation, mice were sacrificed; the tracheas were removed aseptically and homogenized in a glass mortar and pestle in 1 ml of sterile phosphate-buffered saline (pH 7.4).

(ii) Bacterial identification. One-λ (0.001-ml bacteriological loop) portions of tracheal homogenate were plated on both sheep blood agar plates and eosin methylene blue agar plates and cultured aerobically. The number of distinct isolates in quantities of $\geq 10^3$ CFU per trachea was determined by analyzing colony morphology, hemolytic pattern, eosin methylene blue selectivity, fermentation pattern, and Gram stains; these methods did not allow exact identification. We defined colonization as the recovery of any bacterial isolate in concentrations of $\geq 10^3$ CFU per trachea. In some experiments the number of distinct isolates in lung homogenates was also determined.

(iii) Tracheal adherence during spontaneous colonization. In separate groups of mice 7 days post-virus inoculation, the trachea was cannulated near the larynx, and the trachea and distal airways were washed with sterile phosphate-buffered saline solution by injection and withdrawal of a 1.0-ml volume three times. The trachea was then removed and homogenized, and the trachea homogenate and wash fluid were serially diluted and quantitatively cultured. The concentrations of each distinct bacterial isolate in the tracheal homogenate and wash fluid were then compared to estimate the relative adherence to tracheal surface. In these experiments we recovered all the flora present in the trachea and in the lavage fluid and not just isolates in concentrations of $\geq 10^3$ CFU. We used this method to analyze all the bacterial flora in each animal rather than using the group average determinations with washed tracheas described above for the staphylococcal aerosol exposure experiments.

Scanning electron microscopy. Tracheas were excised, rinsed in phosphate-buffered saline (pH 7.4), and fixed in 2.5% phosphate-buffered glutaraldehyde. Osmium tetroxide postfixation was followed by ethanol dehydration and carbon dioxide critical-point drying. The samples were then coated with carbon and gold-palladium and examined in a JEOL 100Cx TEMSCAN.

Data analysis. The results were analyzed by the *t* test, the chi square test, and the Wilcoxon rank sum test (3). A probability of ≤0.05 was considered significant.

RESULTS

Virus titers. In these experiments influenza titers in the tracheal homogenates increased to peak levels on day 5 after virus inoculation and fell to levels undetectable with this assay by day 10 (50% tissue culture infectious dose units per trachea: day 1, $10^{2.2 \pm 0.1}$; day 5, $10^{3.7 \pm 0.3}$; day 7, $10^{2.9 \pm 0.2}$; day 10, $<10^{2.0}$).

Tracheal clearance in influenza-infected mice. We used an aerosol exposure technique to deposit *S. aureus* on the tracheal surface and then measured the change in the number of viable staphylococci over time to determine clearance. For these studies, we removed only the proximal trachea rather than dissecting out the trachea and major bronchi as in our earlier work (13). These experiments demonstrated that staphylococcal clearance from the tracheal surface was significantly delayed in influenza-infected mice (Fig. 1). The number of staphylococci deposited on the proximal trachea during the aerosol exposure was similar in control and influenza-infected mice (control, 84.5 ± 17.5 × 10^2 CFU per trachea; influenza-infected mice, 79.7 ± 14.3 × 10^2 CFU per trachea; $P > 0.05$ by the *t* test), suggesting that influenza infections did not produce a major change in the "adherence" sites for staphylococci in this region of the trachea. We further tested the relative adherence of staphylococci to the tracheal surface by gently washing the trachea after aerosol exposure. The fractions of deposited staphylococci adherent after this washing procedure were similar in control and influenza-infected mice (control mice, 41 ± 16%; influenza-infected mice, 69 ± 19%; $P > 0.05$ by the *t* test). The average number of staphylococci deposited on the proximal tracheas during the aerosol exposure in these latter experiments was again similar in control and influenza-infected mice ($P > 0.05$ by the *t* test).

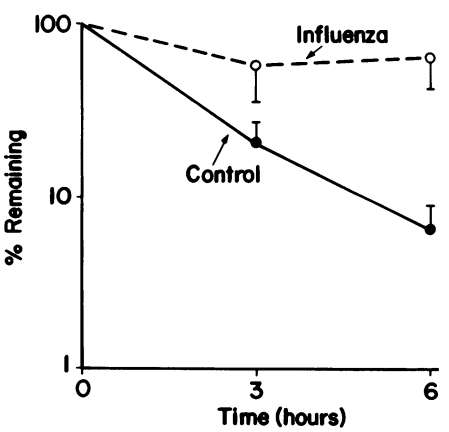

FIG. 1. Staphylococcal clearance from tracheal surfaces. The residual fraction of viable staphylococci in tracheal homogenates is plotted against time. The data points represent the results from 39 control mice (●) and 39 influenza-infected mice (○). The difference at 6 h is statistically significant ($P < 0.01$ by the *t* test).

Spontaneous bacterial colonization of the trachea. In pilot studies with healthy mice chosen randomly from our animal colony during an 8-week period, the majority (seven of nine) had low numbers of gram-positive (GP) isolates (range, 4 to 1,000 CFU) in tracheal homogenates. Only two (22%) had sufficient concentrations of bacteria (i.e., ≥10^3 CFU per trachea) to be considered "colonized" by our definition. In studies with mice sacrificed 1 day after inoculation, 25% (two of eight) of mice receiving diluent and 37.5% (three of eight) of mice receiving virus were colonized ($P > 0.05$ by chi square analysis). These results suggested that brief ether anesthesia and fluid inoculation into the nares did not alter the normal tracheal flora and that subsequent differences between control and experimental mice could be attributed to a virus effect and not aspiration. On days 5, 7, and 10 after inoculation, 25% (5 of 20) of control mice and 56.3% (27 of 48) of influenza-infected mice were colonized with either GP or gram-negative (GN) isolates or both (results pooled from all 3 days in both groups; $P < 0.05$ by chi square analysis). Only 1 control animal had a GN isolate, whereas 13 influenza-infected mice had GN isolates ($P < 0.05$ by chi square analysis). In contrast, there was no difference in GP colonization ($P > 0.05$ by chi square analysis).

Influenza-infected mice also had a significant increase in the average number of isolates per animal (Fig. 2; $P < 0.05$ by the *t* test), and this peaked on day 7 after virus inoculation (Fig. 3). We also determined the average density in the tracheal homogenates by counting the number of colonies of a particular isolate or by estimating the number when there were several isolates with numerous colonies. In control mice there was a mean of 2.0 × 10^3 CFU per trachea, and this increased to 14.1 × 10^3 in influenza-infected mice ($P < 0.05$ by the Wilcoxon rank sum test).

We estimated the relative adherence of the bacterial species which colonized the trachea by lavaging the tracheas with saline before homogenization and then comparing the relative concentrations of distinct isolates in the lavage fluid and in the homogenate. In eight influenza-infected mice sacrificed on day 7 with 25 total isolates, the lavage to trachea ratios ranged from ≤0.001 to 2.23, with the majority less than 0.016 (Fig. 4). There was no difference in apparent adherence

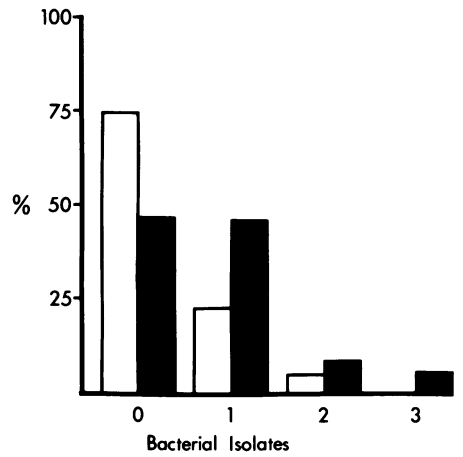

FIG. 2. Histogram of the number of bacterial isolates per animal. The number of distinct isolates in concentrations of $\geq 10^3$ CFU per trachea is plotted for control (open bars) and influenza-infected (black bars) mice (sacrificed on days 5, 7, and 10). Control mice had 0.30 ± 0.12 isolates per animal, and influenza-infected mice had 0.77 ± 0.12 ($P < 0.05$ by the t test).

(lavage to trachea ratios) between GN and GP isolates in these mice. In control mice the predominant isolate was a GP streptococcus which adhered tightly to the trachea (ratio always less than 0.02). These results suggest that the GP bacteria acquired by spontaneous colonization adhere tightly to the tracheal surface in healthy

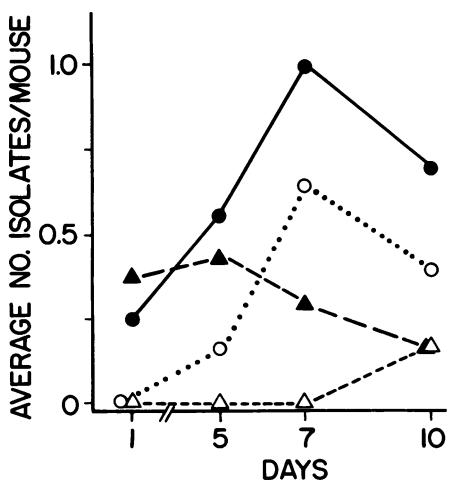

FIG. 3. Average number of bacterial isolates per mouse during influenza infections. The average number of isolates in concentrations of $\geq 10^3$ per animal (calculated from the total number of isolates and total number of mice sacrificed on a particular day after inoculation) peaked in influenza-infected mice on day 7. Symbols: ▲, control mice, total isolates; △, control mice, GN isolates; ●, influenza-infected mice, total isolates; ○, influenza-infected mice, GN isolates.

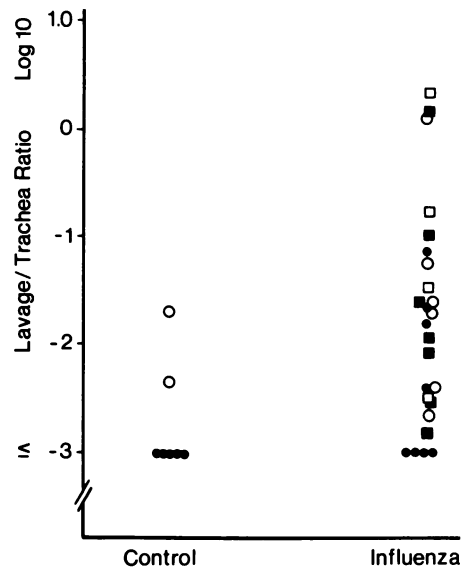

FIG. 4. Relative adherence of colonizing bacteria to tracheal surface. The number of CFU of each bacterial isolate recovered in lavage fluid is compared to the number of CFU of the same isolate in the tracheal homogenate using a ratio (lavage fluid to trachea). Symbols: ○, GP $> 10^3$ CFU in tracheal homogenates; ●, GP $< 10^3$ CFU in tracheal homogenates; ■, GN $\geq 10^3$ CFU in tracheal homogenates; □, GN $< 10^3$ CFU in tracheal homogenates. Ratios of $<10^{-3}$ are included in the −3 level.

mice and that adherence is reduced during influenza infections. The relative infrequency of GN bacteria in control mice precludes any conclusion about GN adherence in influenza-infected mice.

Tetracycline therapy started 2 days after virus inoculation reduced the colonization rate in influenza-infected mice to 15.4% (4 of 26; pooled from days 5, 7, and 10). The average number of isolates (three GP and three GN total) per mouse did not differ significantly from control uninfected mice ($P > 0.05$ by the t test). Separate groups of mice also received amantadine in their drinking water starting 2 days after virus inoculation. Although these animals ingested approximately 6 mg/kg per day, there was no reduction in either the virus titers (data not shown) or the frequency of bacterial colonization (52.4% [11 of 21] with 9 GP isolates and 13 GN isolates).

Resolution of GN colonization. We repeated part of the experiments described above to determine whether or not the GN flora are eventually eliminated from the trachea and to determine whether spontaneous bacterial pneumonia developed in these animals. The mice used in these experiments generally had less dense normal GP flora, and therefore we identified all distinct isolates in these experiments regardless

TABLE 1. Resolution of GN colonization

Mice	No. of mice with GN colonization/ total no. of mice on the following day after virus inoculation:[a]			
	7	10	14	21
Control	0/12	0/12	0/7	0/4
Influenza infected[b]	4/20	5/20	0/12	0/12

[a] In these experiments 100-λ portions of tracheal homogenates were plated.

[b] The differences in the total number of mice with GN colonization between control and influenza-infected mice are significant by chi square analysis ($P < 0.02$).

of their concentration in tracheal homogenates. The results in Table 1 demonstrate that GN colonization again occurred only in influenza-infected mice in this series of experiments and that this resolved by day 14 after virus infection. We cultured lung homogenates from control and influenza-infected mice (days 7 and 10) and did not find significant bacterial growth (defined as $>10^4$ CFU per lung pair) in either control or influenza-infected mice, even though 42.5% (17 of 40) of influenza-infected mice had gross morphological evidence of viral pneumonia.

Scanning electron microscopy. The mucosa in specimens from animals with influenza was fragile and developed linear tears in the end regions during processing. Tracheas from day-2 mice had patchy areas of desquamation (Fig. 5B). By days 5 and 7 there was evidence of regeneration, with multiple areas which had long and short microvilli (Fig. 5C). There were few cells with cilia in these preparations. By day 10 some of the epithelial cells had sparse cilia, but there were still large areas of mucosa with long microvilli (Fig. 5D).

DISCUSSION

These results demonstrate that spontaneous quantitative and qualitative changes in the tracheal flora, including the appearance of GN bacteria, occur during sublethal influenza infec-

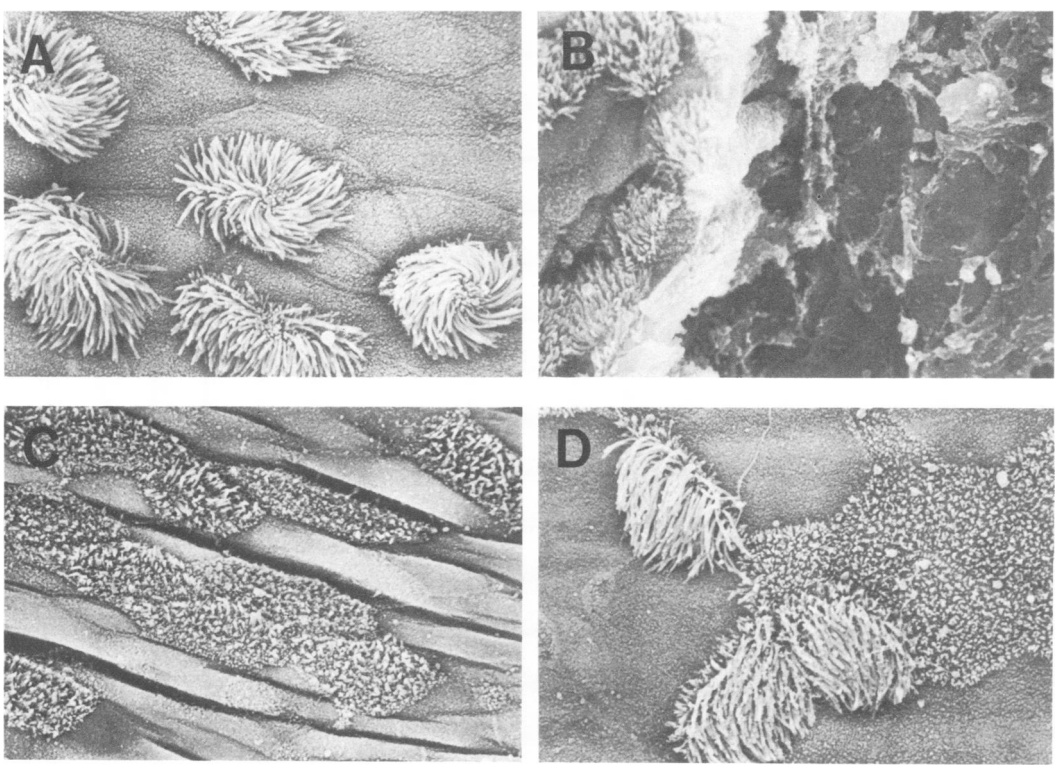

FIG. 5. Tracheal morphology during influenza infection. (A) Trachea from control mice (final magnification, ×2,000). (B) Trachea from an animal sacrificed 2 days after virus inoculation. Note the large circular area of desquamation adjacent to normal mucosa, with damaged mucosa lifting up along the border (final magnification, ×1,500). (C) Trachea from an animal sacrificed 5 days after virus inoculation. Cilia are completely gone, and there are large areas of mucosal cells with numerous long microvilli (final magnification, ×1,500). (D) Trachea from an animal sacrificed 10 days after virus inoculation. Some epithelial cells now have sparse cilia, and many have numerous long microvilli (final magnification, ×2,000).

tions in mice, that these changes persist after viral titers have fallen below detectable levels, and that they are temporally associated with overt damage to the tracheal mucosa. These changes in tracheal flora developed during established influenza infections of the trachea and did not develop after ether anesthesia and fluid inoculation into the nares. Therefore, influenza tracheitis appears to alter tracheal characteristics which limit colonization to "normal" flora and allows other nasopharyngeal flora to colonize the trachea, presumably, after minor aspirations. Ramphal et al. reported that the tracheal mucosa undergoes complete desquamation within 3 days after intranasal inoculation of an attenuated virus and that regeneration begins by 5 days and requires 14 days for complete repair (15). Although the desquamation was less extensive in our mice, we found a similar pattern of injury and prolonged repair. In our mice the patches of mucosa with long microvilli are the most obvious alteration consistently present in the influenza-infected mice with increased colonization. However, Ramphal et al. have demonstrated that *Pseudomonas aerginosa* adhered only to desquamating cells of murine tracheas infected with influenza virus and did not adhere to basal cells or regenerating epithelium (16). Therefore, new colonization appears to occur on an abnormal epithelial mucosa and not on the basal cell layer, but the exact location probably depends on the bacteria.

These changes in tracheal flora may develop because the viral infection or the regeneration process provides new "adherence" sites for colonizing bacteria or because the damaged mucociliary apparatus cannot maintain effective tracheal clearance. Our experiments with an aerosol challenge model demonstrated that influenza infection reduced tracheal clearance processes but did not appear to alter the adherence of staphylococci to the tracheal surface. In addition, the normal GP flora in influenza-infected mice appeared less adherent to the tracheal surface than the GP flora found in control mice. However, these conclusions regarding in vivo adherence differ somewhat from results obtained with in vitro models. Davison and Sanford (2) have demonstrated that staphylococci have increased adherence to influenza-infected Madin-Darby canine kidney cells, and Selinger et al. (18) have demonstrated that staphylococci initially have increased adherence to epithelial cell lines infected in vitro with influenza virus but that by day 9 after infection adherence is actually reduced. Therefore, these in vitro studies suggest that viral infections alter the cell surface and increase either the number or the affinity of bacterial binding sites and thereby increase bacterial adherence. However, this change in adherence seems to depend on the stage of viral infection. We should note that our studies utilized one mouse-adapted virulent virus and evaluated only proximal tracheal function. It is possible that influenza infections in the distal tracheobronchial tree or with different viruses may produce greater changes in the adherence capacity of the mucosal surface (21).

Other investigators have described spontaneous changes in the lower respiratory tract flora during murine infections with respiratory viruses (8). Sellers et al. reported that 40% of influenza-infected mice had superinfections with GN bacilli which produced a purulent bronchopneumonia distinct from the background viral pneumonitis (19). Yealland and Heath also recovered a coliform organism from influenza-infected mice but did not think that these isolates had any effect on the pneumonic process or mortality (22). The tracheal isolates we recovered did not appear to be pathogenic either, since none of 40 mice sacrificed on days 7 and 10 after virus infection contained large numbers of bacteria in lung homogenates. Therefore, the significance of bacterial colonization clearly depends on the bacterial virulence.

Acute viral infections also appear to alter the flora in the upper and lower respiratory tract in both healthy adults and patients with chronic lung diseases (7, 22). In patients with chronic obstructive pulmonary disease, prospective studies have demonstrated both qualitative and quantitative changes in respiratory tract flora during acute exacerbations (4), and in several studies acute exacerbations in these patients have been associated with concurrent viral infections (6, 20). Overall, these studies in humans also suggest that viral infections can alter the population dynamics of the established flora in the nasopharynx and airways or allow colonization with new bacteria after an appropriate exposure. Our experiments with tetracycline therapy suggest that antibiotics are effective even in the presence of viral tracheitis and support the frequent usage of antibiotics in patients with chronic lung diseases during acute exacerbations.

In summary, spontaneous changes in the tracheal flora of mice occur during acute influenza infections, and these changes appear to reflect alterations in tracheal clearance processes and not the appearance of new adherence sites on the tracheal surface. Transient fluctuations in bacterial populations in major airways during influenza infections increase the potential for bacterial deposition in lung parenchyma. Since concentrated inocula of *S. aureus* (and presumably other bacteria) can overwhelm regional defense processes even in the normal lung (14), it seems possible that viral tracheitis could contribute to the development of bacterial pneumo-

nia regardless of the clearance capacity of the underlying lung parenchyma.

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